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When the Nose Must Remain Responsive: Glutathione Conjugation of the Mammary Pheromone in the Newborn Rabbit

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Abstract

In insects, xenobiotic-metabolizing enzymes were demonstrated to regulate pheromones inactivation, clearing them from the olfactory periphery and keeping receptors ready for stimulation renewal. Here, we investigate whether similar processes could occur in mammals, focusing on the pheromonal communication between female rabbits and their newborns. Lactating rabbits emit in their milk a volatile aldehyde, 2-methylbut-2-enal, that elicits searching-grasping in neonates; called the mammary pheromone (MP), it is critical for pups which are constrained to find nipples within the 5 min of daily nursing. For newborns, it is thus essential to remain sensitive to this odorant during the whole nursing period to display several actions of sucking. Here, we show that the MP is enzymatically conjugated to glutathione in newborn olfactory epithelium (OE), in accordance with the high mRNA expression of glutathione transferases evidenced by quantitative reverse transcription–PCR. This activity in the nose is higher than in the liver and in OE of newborns compared with weanlings (no more responsive to the pheromone). Therefore, the results pinpoint the existence of a high level of MP-glutathione conjugation activity in the OE of young rabbits, especially in the developmental window where the perceptual sensitivity toward the MP is crucial for survival.

Key words: glutathione transferases, mammary pheromone, newborn, nursing, olfaction, perireceptor events, rabbit (*Oryctolagus cuniculus*), xenobiotic-metabolizing enzymes

Introduction

In animals, olfaction is a critical sense allowing for instance fast identification of conspecifics, predators, or food. The olfactory process is sequential, starting from the peripheral detection of odorants, followed by transduction into action potentials relayed into higher level processing structures, and ending by integration in the central nervous system and eventually by motor action. The most peripheral events take place in the olfactory epithelium (OE), where the detection of odorants relies on the olfactory receptor proteins located on the cilia of olfactory sensory neurons. It is now well admitted that perireceptor mechanisms participate in the processing of the ligand before or after its binding to the receptor and modulate final perception. For example, odorant-binding proteins, which are present in the olfactory mucus, have been involved in ligand capture, transport, and presentation to the olfactory receptor proteins (Kaissling 2009; Zhou 2010). However, the olfactory mucus needs to be protected against saturation by various and numerous ligands. Multiple physiological and biochemical processes are at work to rapidly renew or clear the mucus from excess ligands and to keep the immediate environment of olfactory sensory neurons optimally functional. Among these processes, there is growing evidence that enzymatic conversion of odorants into nonodorous metabolites is critical in the modulation of olfactory detection (for review, see Heydel et al. 2013). The enzymes involved in this mechanism belong to the family of xenobiotic-metabolizing enzymes that are devoted to eliminate potentially toxic molecules of exogenous (food,

drugs, pollutants…) or endogenous (hormones, bile acids…) origins in the whole organism. These enzymes belong to a network of proteins acting serially [phase I (functionalization, synthesis of polar metabolite): e.g., cytochrome P450 (CYP); phase II (conjugation, synthesis of hydrophilic metabolite): e.g., glutathione transferases (GST) or UDP-glucuronosyltransferases (UGT); phase III (metabolite cellular excretion): e.g., p-glycoprotein or multidrug resistance transporters]. Different xenobiotic-metabolizing enzymes have been specifically or preferentially detected in the OE (Nef et al. 1989; Ding et al. 1991; Lazard et al. 1991; Ben-Arie et al. 1993; Tamura et al. 1998; Jedlitschky et al. 1999; Heydel et al. 2001; Kurosaki et al. 2004; Zhuo et al. 2004; Thiebaud et al. 2010, 2011). Their function has been investigated in olfaction and it has been hypothesized that they could potentially influence odor detection by: 1) inactivating the signal through degradation or biotransformation, 2) eliminating the signal to limit or avoid receptor saturation, and 3) synthesizing metabolites that become potentially able to interact with olfactory receptor proteins (for review, see Heydel et al. 2013).

The involvement of perireceptor enzymatic conversion in olfactory perception has been studied in insects, especially regarding pheromones. For example, in the male beetle *Phyllopertha diversa*, the inhibition of antennae CYP activity with metyrapone (a nonspecific CYP inhibitor) was correlated with transient anosmia (measured by electrophysiological recording) toward an alkaloid pheromone (Maïbèche-Coisne et al. 2004), suggesting saturation of the olfactory receptors due to the accumulation of the pheromone. Such a role in pheromonal signal termination was also investigated in *Drosophila melanogaster* regarding the activity of carboxylesterase-6 toward the cis-vaccenyl acetate pheromone (Chertemps et al. 2012). Moreover, male sphinx moth *Manduca sexta* expresses GST specifically in the sex-pheromone–sensitive olfactory sensilla (Rogers et al. 1999). This tissue presents a strong activity of glutathione conjugation toward aldehydes, which are the preferential substrates of GST and the major components of the *M. sexta* pheromone mixture.

So far, no study regarding the olfactory metabolism of pheromones has been conducted in mammals. Much like in insects, these chemostimuli have a critical role in mammalian biology (Beauchamp et al. 1976; Johansson and Jones 2007; Tirindelli et al. 2009). The detection sensitivity of a pheromone must be adequately functional during the period of the vital cycle when it influences the behavior of the animal which is the receiver. Accordingly, as was previously shown in insects, it is reasonable to expect that metabolic processes within the OE do control mammalian pheromone bioavailability.

The present study focused on the well-defined mammalian model of pheromone communication occurring between the female European rabbit, *Oryctolagus cuniculus*, and her offsprings. In this species, newborns have only 5 min per day to get the amount of milk that will ensure their initial survival and growth (Zarrow et al. 1965); missing more than one sucking episode severely jeopardizes survival (Coureaud et al. 2000). These altricial newborns can only rely on olfaction to localize and grasp nipples as immediately as possible when the female becomes available. Thus, they display searching actions in contact with the maternal abdomen triggered by odor cues emitted by females into their milk and on nipples (Schley 1979; Hudson and Distel 1983; Coureaud et al. 2001; Moncomble et al. 2005). Among these cues, some are learned before and/or after birth, but at least one signal is spontaneously active at its very first postnatal presentation (reviewed in Coureaud, Fortun-Lamothe, et al. 2008; Schaal et al. 2008; Coureaud et al. 2010). This volatile compound, identified as 2-methylbut-2-enal (2MB2), is able to elicit typical orocephalic movements related to sucking in more than 90% of the pups (Coureaud 2001; Coureaud et al. 2003; Schaal et al. 2003). It qualifies as a pheromone as it satisfies to the stringent definitional criteria proposed for mammalian pheromones (Beauchamp et al. 1976). Accordingly, this monomolecular signal has been named "the mammary pheromone" (MP) (Coureaud 2001; Coureaud et al. 2003; Schaal et al. 2003). As other mammary cues of lactating rabbit females (Hudson and Distel 1986), the MP appears to be processed by the main olfactory system, not by the vomeronasal system (Saucier et al. 2005; Charra et al. 2012). Its perception by pups is concentration dependent (Coureaud et al. 2004) and changes over time, in that orocephalic responsiveness of pups is highest during the first 10 days following birth before to progressively decrease and completely vanish at weaning (Coureaud, Langlois, et al. 2006; Coureaud, Rödel, et al. 2008). Neonatal ability to detect the MP has been shown to be vital, as those individuals who do not react to it on postnatal day 1 die before weaning (Coureaud et al. 2007). In addition to its releasing properties, the MP efficiently promotes learning of any novel odor that is associated with it (Coureaud, Moncomble, et al. 2006; Coureaud et al. 2010). To sum up, it is essential for rabbit pups to detect the MP in the temporally restricted and highly competitive conditions that they face during nursing. It is important to note that rabbit pups do not select and attach to a single nipple, as their nursing behavior is organized sequentially in searching for one nipple, shortly sucking it, and then swapping to the same or to another nipple (randomly chosen) previously sucked by another pup. There is no teat order in the rabbit, that is, no spontaneous preference for, nor spatial learning of, a given nipple or a sequence of successive nipples, at least early in lactation (Hudson and Distel 1982, 1983; Bautista et al. 2005, 2008; Coureaud, Fortun-Lamothe, et al. 2008). Thus, during their interaction with the mother to suck, newborn rabbits do not need to detect the MP only once to attach to a single nipple, but several times over several nipples to get their share of milk (one may thus suggest that newborn rabbits perceive a similar level of MP contained in milk ejected from the distinct nipples).

Therefore, the neonatal MP detection mechanism has to be ready for successive cycles of milk intake within the 5-min opportunity of daily sucking and to maintain the highest level of MP receptivity at the level of olfactory receptors.

2MB2 is an α,β-unsaturated aldehyde which belongs to alkenals, a family of chemicals produced from the free radical oxidation of endogenous fatty acids or environmental pollutants (O'Brien et al. 2005). Their toxic properties have been widely studied (Griffin and Segall 1986; Janzowski et al. 2003; Alhamdani et al. 2006). Some alkenals have been characterized as pheromones in insects (Riddiford 1967; Yamakawa et al. 2011). Their toxicology has been widely studied (Griffin and Segall 1986; Janzowski et al. 2003; Alhamdani et al. 2006). The catabolism of alkenals mobilizes glutathione conjugation and involves GST (Berhane et al. 1994). GST constitutes a superfamily of xenobiotic-metabolizing enzymes localized in cytosol or microsomes. They catalyse the conjugation of the tripeptide reduced glutathione (GSH, γGlu-Cys-Gly) to electrophilic substrates from exogenous or endogenous origin (Hayes et al. 2005). Numerous isoforms of these enzymes have been isolated both in the OE and vomeronasal organ of rodents (Ben-Arie et al. 1993; Banger et al. 1994; Krishna et al. 1994; Green et al. 2005) but have not yet been investigated in lagomorphs. In particular, an isoform has been evidenced in rat and mouse OE, presenting a preferential and zonal expression of GST (lateral olfactory turbinates), suggesting that these enzymes might be involved in the modulation of olfactory detection (Ben-Arie et al. 1993; Whitby-Logan et al. 2004).

In the present study, we focused on the glutathione conjugation (phase II metabolism) potentially directed toward the MP in the OE of newborn rabbits. According to the hypothesis that this activity would be of importance to maintain a high olfactory sensitivity toward this signal at birth, we made different comparisons: 1) OE versus liver which is the main metabolic organ, 2) newborns versus weanlings in each tissue, since the activity of the MP as a behavioral releaser decreases along early development; and 3) age and tissue comparison toward other aldehydes perceived as common odor cues (in terms of behavioral reactions) and toward a GST (nonaldehydic) reference substrate. Tissue and age dissociations were also applied to the mRNA expression of the olfactory enzymes potentially involved in glutathione conjugation activity.

Materials and methods

Animals

New Zealand rabbits (Charles River strain) originated from the breeding colony of the Centre de Zootechnie (Université de Bourgogne, Dijon). Adult females and males were housed in individual cages and kept under a constant 12:12–h light:dark cycle (light on at 7:00 AM) with ambient air temperature maintained at 21–22 °C. Water and food (Lapin Elevage #110, Safe, France) were provided ad libitum. Two days before the expected day of parturition, a nest box (0.39 × 0.25 × 0.32 m) was fixed to the cages of pregnant females. The day of birth was designated as postnatal day 0. To even out pup–female interaction, females' access to the nest was allowed for 15 min per day at 11:30 AM (i.e., mimicking the short daily nursing episode displayed by rabbit females; Zarrow et al. 1965). We used a total of thirty 1- or 2-day-old newborn rabbits (from 15 litters), thirty 30-day-old weaned animals (weaning occurred on day 28; 15 litters) for metabolism assays, and one hundred twenty-five 2-day-old pups (from 25 litters) for testing behavioral responsiveness to the MP and other aldehydes. The local, institutional, and national rules (French Ministries of Agriculture and of Research and Technology) regarding the care and experimental use of the animals were followed. Thus, all experiments were conducted in accordance with ethical rules enforced by French law and were approved by the Ethical Committee for Animal Experimentation (Dijon, France; protocol no. 3507).

Tissue sampling

After decapitation of the animal, the liver and OE were immediately removed, placed into a sterile phosphate-buffered saline (PBS 0.05 M, pH 7.4), frozen in liquid nitrogen for tissue homogenate or placed into a RNAse-free vial to be immediately stored at −80 °C. Careful OE dissection was performed to avoid contamination with respiratory epithelium.

mRNA extraction and real-time reverse transcription–PCR

RNAs were extracted from rabbit tissues using Trizol reagent (Invitrogen) and TissueLyser (Qiagen) for 2 × 1 min at 30 Hz and then treated with RNase-free DNase to avoid contamination by genomic DNA. Total RNA (1 µg) was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad). cDNA were then diluted 12.5 times in RNase-free water. Real-time PCR reactions were carried out on a MyIQ real time detection system (BioRad) using the IQ SYBR Green Supermix (BioRad), with 12.5 µL of Supermix, 1 µL of sense and 1 µL antisense primers (or 1.5 µL each for GST Pi "like" primers), and RNase-free H_2O for a final volume of 20 µL, then 5 µL of diluted cDNA was added to each reaction. The real-time PCR conditions were as follows: 95 °C for 30 s to activate the DNA polymerase, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each reaction was performed in triplicate and the mean of the three independent biological replicates (corresponding to three extractions) was calculated.

Tissue homogenates

Livers and OE were homogenized in PBS using a TissueLyser (Qiagen) for 2 × 1 min at 30 Hz. The tissue homogenates were

centrifuged for 5 min at 4 °C and 12000 × *g*. The supernatant homogenates corresponding to 8–10 animals were pooled (1 pool per assay; *n* = 3) and the protein content was measured by the technique of Lowry et al. (1951) using bovine serum albumin as standard. Small aliquots of supernatant (200 µL) containing GST were stored at −20 °C.

Enzymatic glutathione conjugation assay toward the MP and analysis by thin-layer chromatography

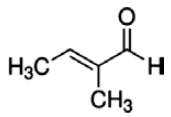
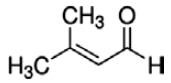
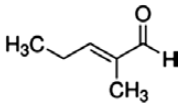
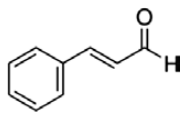
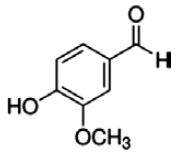
GST conjugation activity toward the MP was determined using the thin-layer chromatography (TLC) assay described by Ben-Arie et al. (1993). A 50-µL reaction mixture containing 1 M 2MB2 (MP; Sigma- Aldrich), 0.1 M glutathione (Sigma), and OE homogenate (10 µg/µL total proteins) was incubated in 100 mM phosphate buffer at 37 °C for 1 h. The reaction was stopped with 5 µL of $CuSO_4$ (1 M; VWR). To visualize the reaction products, 4 µL of reaction supernatant sample (75000 × *g*, 5 min) was loaded onto 0.25-mm TLC cellulose-coated glass plates (20 × 20 cm; Merk), developed using butanol:acetic acid:water (12:3:5 by volume) and stained with ninhydrin (0.25% w/v in acetone; 37 °C).

Supernatant sample was also loaded onto a column containing cellulose equivalent to that used in TLC (0.25 mm) and was eluted using butanol:acetic acid:water (12:3:5 by volume). Fractions containing MP-glutathione conjugate were identified by TLC and used for mass spectrometry analysis. Structural characterization of glutathione and MP-glutathione conjugate was performed using an Esquire LC ion trap mass spectrometer (Bruker Daltonique). Sample was directly introduced by infusion at a flow rate of 200 µL/h into the electrospray ionization (ESI) source. Molecules were ionized in negative ESI (i.e., ESI−) mode with a capillary voltage of 3500 V and nebulizer gas (N_2 temperature of 70 °C) at a flow rate of 5 L/min. Ion trap parameters were set to −40 and −5 V for the 2 skimmers, trap drive equal to 30.0 (arbitrary units), and the ion trap scanned from 50 to 800 m/z in 5 ms.

Enzymatic glutathione conjugation assay toward the MP and other aldehydes and quantitative analysis of the glutathione conjugates by high-performance liquid chromatography

Incubations were carried out in a system containing 30 µg/µL of total proteins of tissues supernatant homogenates in PBS (except for newborn OE, ca. 18 µg/µL of proteins), 15 mM reduced glutathione (Sigma-Aldrich) dissolved in double distilled water, and 300 mM aldehydes presented in Table 1 (Sigma-Aldrich) dissolved in absolute ethanol. The final incubation volume was 50 µL. After 80 min incubation at 37 °C, the reaction was stopped with 50 µL of a $CuSO_4$ solution (25%) followed by centrifugation for 3 × 10 min at 4 °C and 13 500 × *g*. Supernatant containing glutathione-aldehyde

Table 1 Total versus nonenzymatic glutathione conjugation and % of responding newborn rabbits to several aldehydes, including the MP

Tested aldehydes	Total/nonenzymatic conjugation	% of newborn rabbit responsiveness (*n*)
2-methylbut-2-enal (MP) 	2.36**	92 (25)
3-methylbut-2-enal 	1.93**	28.75 (35)
2-methyl-2-pentenal 	0.97 (ns)	4 (25)
Cinnamaldehyde 	4.13**	0 (20)
Vanillin 	None	0 (20)

For glutathione conjugation experiments, *n* pups tested = 5. For testing of behavioral responsiveness, *n* of pups = 20–35 pups/odorants (see the text for comparison results). ns, no significant differences; none, no glutathione conjugation (Student's *t*-test).
**$P < 0.01$.

conjugates were analyzed by a high-performance liquid chromatography (HPLC) method described below.

The reversed phase HPLC of glutathione-aldehyde conjugates was performed on C18 reversed phase column (NUCLEODUR 100–5 C_{18} ec, 4.6 × 250 mm, Macherey-Nagel) using a multistep gradient with (A) 0.1% trifluoroacetic acid (TFA) in methanol and (B) 0.1% TFA in water as mobile phase. Gradient elution began at 0.5% (A) and 99.5% (B), was kept constant for 20 min, increased to reach 40% (A) and 60% (B) at 40 min, kept constant for 10 min, and then reduced to reach 0.5% (A) and 99.5% (B) at 60 min during 10 min. The total analysis time was 70 min. Ten microliters of samples was injected in the HPLC system Ultimate 3000 VWD (Dionex). The flow rate of the mobile phase was set at 0.5 mL/min, and the column was maintained at 25 ± 0.1 °C. The effluent from the HPLC column was mixed with a postcolumn reagent solution containing 2% ninhydrin dissolved in methanol using a T-type mixing device at a flow rate of 0.5 mL/min. The mixture was then passed through a 2.5 ml reaction coil immersed in an 80 °C column oven (965-CO, Jasco) and UV/VIS detector set to 570 nm. For investigations concerning the age- and tissue-related variations, reduced glutathione concentrations, aldehyde concentrations, and OE or liver concentrations were tested to determine optimal conditions allowing to obtain maximal area within the chromatograms. Peak area measurements corresponding to glutathione-conjugated aldehydes were normalized to protein content of tissue homogenate (mAU/min/mg protein). These results were additionally normalized to the measurements performed in absence of homogenate (nonenzymatic conjugation) to express enzymatic conjugation.

Enzymatic glutathione conjugation assay toward 1-chloro-2,4-dinitrobenzene

1-Chloro-2,4-dinitrobenzene (CDNB) is a reference substrate for measuring GST activities (Habig and Jakoby 1981). Incubations were carried out in a system containing 3 µg/µL of protein of cleared tissue extract in PBS, 5 mM reduced glutathione dissolved in double distilled water and 10 mM CDNB dissolved in PBS. The final incubation volume was 15 µL. After 60 min incubation at 37 °C, the supernatant fraction was diluted 1:2 in PBS and centrifuged for 3 × 10 min at 4 °C and 13 500 × *g*. All samples were diluted 1:5 in PBS before analysis. Supernatant fraction containing glutathione-CDNB conjugate was analyzed by spectrophotometry at 340 nm using NanoDrop ND-1000 (NanoDrop Technologies). All samples were diluted 1:5 in PBS before analysis. For investigations concerning the age- and tissue-related variations, reduced glutathione concentrations, CDNB concentration, and OE or liver concentrations were tested to determine optimal conditions allowing to obtain maximal optical density.

Control stimuli

Classical aldehydic odorants were incubated in absence or presence of newborn rabbit OE, as described above, to determine the proportion of their spontaneous conjugation with glutathione versus enzymatic conjugation, in comparison with the MP. These aldehydes included 3-methylbut-2-enal, 2-methyl-2-pentenal, and cinnamaldehyde (Sigma-Aldrich). Vanillin (Sigma-Aldrich), an aldehyde known to be metabolized by another metabolic pathway than glutathione conjugation (Panoutsopoulos and Beedham 2005), was also tested as a negative control.

Behavioral testing

Rabbit pups were individually tested with all above odorants through a previously validated procedure to assess the degree of behavioral activity of the stimuli (Coureaud et al. 2003, Schaal et al. 2003; Coureaud et al. 2004; Coureaud, Langlois, et al. 2006; Coureaud, Rödel, et al. 2008). The test consists in a 10-s presentation of a glass rod carrying the stimulus (20 cm long, 0.4 cm in diameter) right under the pup nares. A stimulus was considered inactive when it did not release head-searching and oral grasping movements. When only a sniffing response was elicited, the stimulus was considered neutral.

To minimize litter effects, each experimental group was drawn from 4 or 5 litters, with a maximum of 5 pups tested per litter in a given group. A group was tested either with pure MP only (10^{-6} g/mL, diluted in water; n = 25 pups) or to another aldehyde (10^{-6} g/mL, diluted in water) followed by exposure to the MP (intertrial interval: 120 s; n = 20–35 pups/group depending on the aldehyde; Table 1). If a pup responded to a stimulus and touched the glass rod, its muzzle was softly dried before the next stimulation. The pups were immediately reintroduced to the nest after testing.

Statistical analyses

For the real-time PCR data, all results were normalized to the 18S and GAPDH mRNA level and calculated using the $\Delta\Delta C_t$ method (Pfaffl et al. 2002). Then transcript level ratios were compared between ages (or tissue fractions) using the Relative Expression Software Tool (REST, REST-MCS beta software version 2) with 2000 iterations. This is based on the probability of an effect as large as that occurring under the null hypothesis (no effect of the treatment), using a randomization test (Pair Wise Fixed Reallocation Randomisation Test) (Pfaffl et al. 2002). For the glutathione conjugation assay, data were analyzed using the Student's *t*-test to compare the activity in the different tissues. Data are expressed as means ± standard error of the mean and normalized by protein concentration. Finally, regarding behavioral responsiveness, the frequencies of pups responding to the MP and other aldehydes were compared using the Pearson's χ^2 test

when the data were independent (i.e., independent groups of pups tested to the same stimuli), with Yates correction when necessary, and the McNemar test when the data were dependent (same pups tested for their response to distinct stimuli).

Results

Glutathione conjugation of the MP in the newborn rabbit OE

To investigate the potential glutathione conjugation of the MP, the molecule was incubated with reduced glutathione and a homogenate of rabbit pup OE containing the enzymes putatively involved in the conjugation process. Control samples omitted either MP or tissue homogenates. TLC on the control without homogenate showed a ninhydrin-stained band (Figure 1A) corresponding to the glutathione, also visible on the 2 other spot separations. The full component reaction showed an extra band when compared with the control without MP (Figure 1A). The chromatographic fraction corresponding to this band was analyzed by mass spectrometry and identified as a glutathione conjugate of the MP [MP m/z (84) + glutathione m/z (307) = m/z (391)] (Figure 1B).

Glutathione conjugation of different aldehydes in newborns OE and corresponding behavioral properties

Glutathione conjugation is a phase II pathway involved in the detoxification of potentially toxic xenobiotic compounds, such as aldehydes. Glutathione is a nucleophilic chemical that can spontaneously bind to aldehydes, but this reaction is strongly accelerated in presence of the GST enzymes. For this reason, we compared the total conjugation activity versus the spontaneous chemical conjugation for different aldehydes including the MP (Table 1).

Conjugation activity toward the aldehydes was analyzed with regards to their behavioral properties. Differences occurred regarding the responsiveness of pups to the stimuli ($\chi^2 = 74.8$, df = 4, $P < 0.001$). Among the 25 pups tested to the MP only, 92% displayed a positive response. Regarding the other selected aldehydes, 3-methylbut-2-enal (3MB2) was behaviorally active but at a much lower level than the MP (28.75% of responding pups; 3MB2 vs. MP: $\chi^2 = 23.7$, $P < 0.001$; 3MB2 compared with the 3 other aldehydes: $\chi^2 > 4.35$, $P < 0.03$) (Table 1). Interestingly, the MP and 3MB2 presented an important total/nonenzymatic conjugation ratio (2.36 and 1.93, respectively) meaning that their conjugation depends mainly on GST catalytical activity (Table 1). Conversely, both 2-methyl-2-pentenal and cinnamaldehyde presented nonsignificant behavioral activity. However, the first exhibited a low total/nonenzymatic conjugation ratio (close to 1), whereas the second showed the highest ratio (4.13). One may note that in pups which did not or weakly respond to aldehydes other than the MP, the low responsiveness was not general since they strongly responded to the MP itself (> 91%; McNemar tests: $\chi^2 > 16$, $P < 0.001$ for all 2×2 comparisons).

Since cinnamaldehyde presented a high total/nonenzymatic ratio, it has been used for other comparisons (tissue

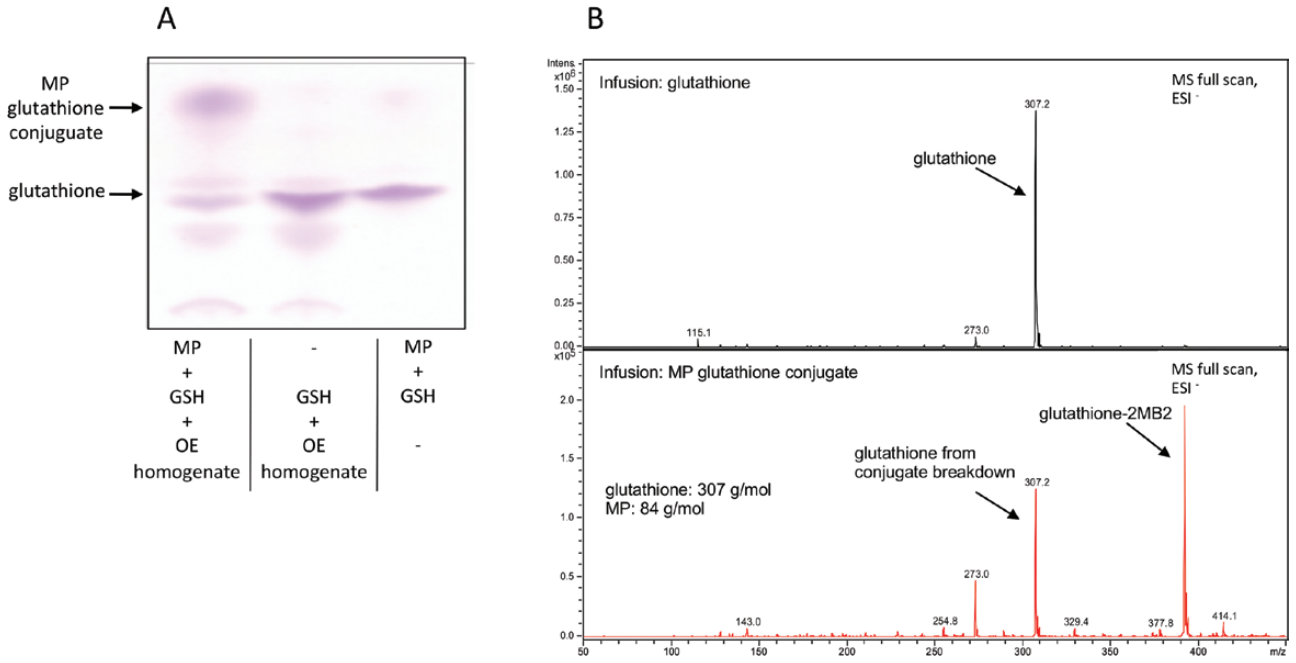


Figure 1 Glutathione conjugation of the MP in the OE of newborn rabbits. (**A**) TLC showing MP-glutathione conjugation. Controls in lanes 2 and 3 omit either MP or tissue homogenate. The higher band in lane 1 corresponds to the MP-glutathione conjugate. (**B**) Mass spectrometry analysis of glutathione (top) and MP-glutathione conjugate separated on TLC (bottom).

and age) in addition to 3MB2. Concerning the vanillin, no glutathione conjugation activity was detected, as expected.

Tissue- and age-related variations of glutathione conjugation activity toward the MP: comparison with other aldehydes and CDNB

MP-glutathione conjugation activity

Glutathione conjugation activity toward the MP was measured by postcolumn ninhydrin derivatization HPLC method in OE versus liver of neonates and in newborn versus weaned rabbits. The MP conjugation in OE was 2.5 higher in neonates than in older animals (Student's *t*-test, $P < 0.001$; Figure 2A). Moreover, in newborns, the MP-glutathione conjugation activity was 2 times higher in the OE than in the liver, the main site of metabolic activity (Student's *t*-test, $P = 0.014$). The liver of weaned rabbits exhibited a slight (×1.5) but significantly higher conjugation activity toward the MP compared to the liver of neonates (*t*-test, $P < 0.05$).

Comparison with 3MB2 and cinnamaldehyde

The glutathione conjugation activity of 3MB2 and cinnamaldehyde, odorants which released behavioral responses in about 30 and 0% of rabbit pups, respectively, was compared with that of the MP. Glutathione conjugation activity toward these aldehydes was significantly higher in the newborn OE relative to the newborn liver or relative to the OE and liver of weaned rabbits (Student's *t*-test; $P < 0.05$) (Figure 2B,C). Interestingly, regarding the OE, a lower difference between newborn and weaned rabbits appeared for both 3MB2 and cinnamaldehyde (around 30% less) than that observed for the MP (Figure 2B,C vs. Figure 2A). Besides, no difference depending on the age was observed for 3MB2 in the liver, while a significant difference was observed for cinnamaldehyde.

Comparison with CDNB

Interestingly, and comparatively with the MP, glutathione conjugation activity toward CDNB, a non-aldehydic reference substrate of GSTs, presented significant but slight differences (×1.1) between the OE of newborns and of weaned rabbits (*t*-test, $P < 0.01$) and no significant difference between the OE of newborns and liver of weanlings. Unlike with the MP, this activity was higher in the liver compared to the OE of weaned rabbits (*t*-test, $P < 0.01$; Figure 2D).

Tissue- and age-related variations of the GST mRNA expression

Because no GST was previously identified in rabbit OE, specific PCR primers were designed from sequences available in GenBank and coding for GST in different tissues (cerebellum, liver, lung). The primers for the GST Pi were designed from a short EST (expressed sequence tag) sequence assigned as Pi in GenBank; because it matches with Pi in other species, it will be called GST Pi "like" in this work. We detected the 4 corresponding amplification products from newborn OE extracted RNA (average PCR threshold cycle for the different GST alpha, Pi like, microsomal and mu were 20, 24.2, 23.8, and 24.7, respectively). Real-time PCR was performed to compare the mRNA expression of the different GST in OE and liver of neonates and for each tissue between newborn and weaned rabbits (Figure 3). Although tendency in variations can be observed for all GST enzymes, in OE, only GST alpha showed a significantly lower expression in weaned compared with newborn animals ($P < 0.05$). Interestingly, all the enzymes were more expressed in the OE than the liver of neonates ($P < 0.01$). A significantly higher expression was also observed in the OE than the liver of weaned rabbits for GST alpha ($P < 0.05$) and GST Pi like ($P < 0.01$); however, GST microsomal and GST mu did not show significant variation of expression level in the liver after weaning. Except for GST Pi like (no difference, $P > 0.5$), the expression of GSTs was higher in the liver of weaned compared with the liver of newborn rabbits ($P < 0.01$, in all cases).

Orthology analysis

Inparanoid algorithm was used to find orthologous GST isozymes (in rat, mouse, and human) corresponding to the rabbit GST mRNA of this study (Table 2) (Remm et al. 2001). The analysis confirms the classes alpha, mu, pi, microsomal of the GST amplified by RT–PCR in our work and strongly suggests that they respectively correspond to the isosymes GSTA3, GSTM2, GSTP1, and MGST3.

Discussion

Several recent studies have attempted to clarify the role of xenobiotic-metabolizing enzymes in the most peripheral processes leading to olfactory perception in mammals. Thus, when a mixture of odorant and CYP inhibitor was presented to a panel of human adults, their detection of the odorant was altered, suggesting perceptual changes in terms of quality or intensity of that odorant (Schilling et al. 2010). In the mouse, the olfactory mucus converted odorants carrying aldehyde or ester functional groups into their respective acids or alcohols (Nagashima and Touhara 2010). In addition, a carboxylesterase inhibitor administered *in vivo* modified glomerular activation pattern elicited by an odorant metabolized by these enzymes and affected behavioral discrimination (Nagashima and Touhara 2010). We recently brought further input into the field showing that xenobiotic-metabolizing enzymes were involved in olfactory signal termination in the rat, and that CYP activity was necessary to avoid saturation of the

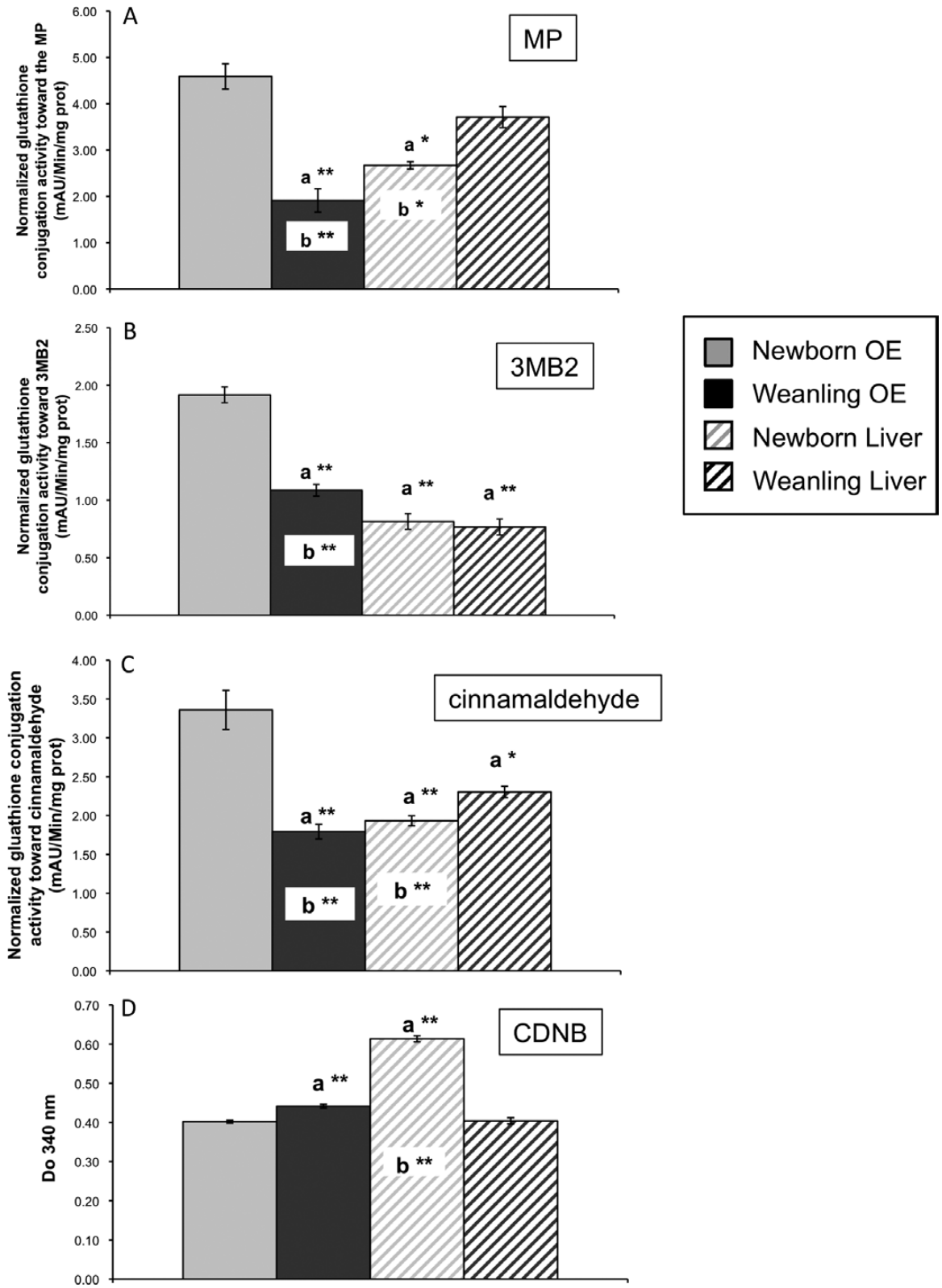


Figure 2 Bar graphs representing the variations of MP (**A**), 3MB2 (**B**), cinnamaldehyde (**C**), and CDNB (**D**) glutathione conjugation activity according to the age of the animals (newborn vs. weaned rabbits) and the tissue (OE vs. liver). HPLC measurements were normalized to protein content of tissue homogenate (mAU/min/mg protein) and to the measurements performed in absence of homogenate (nonenzymatic conjugation) to express enzymatic conjugation. Values are mean ± standard error of the mean from 3 independent assays (pool of tissue from 8–10 rabbits per assay). The significant differences are noted (a) for a comparison with newborn OE and (b) with weanling liver. $*P < 0.05$, $**P < 0.01$. (Student's *t*-test).

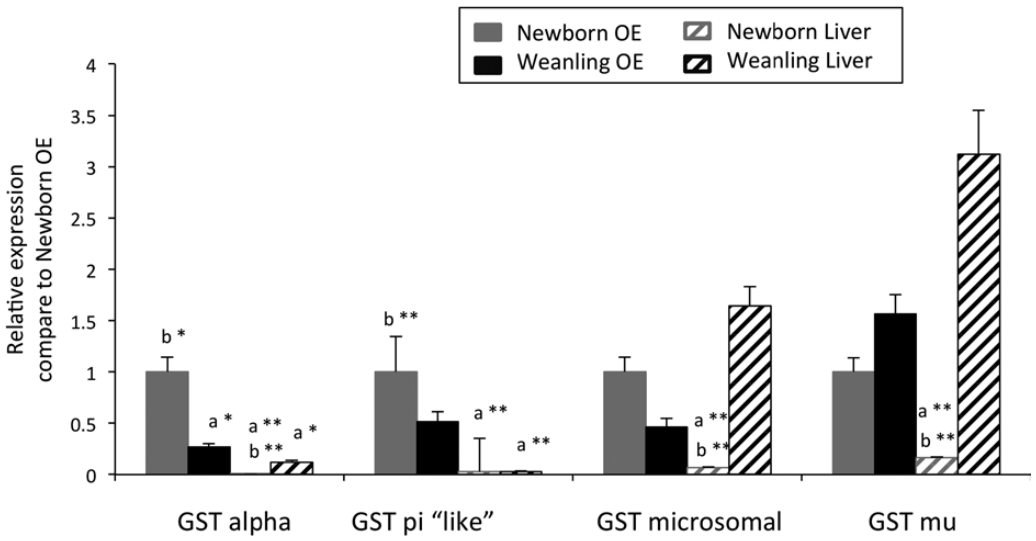


Figure 3 Age and tissue mRNA variations of different GST. RT–PCR was performed to measure the mRNA expression level of GST alpha, the putative form of GST Pi (GST Pi "like"), GST microsomal, and GST mu. The histograms show the relative transcript expression of various xenobiotic-metabolizing enzymes in the OE (filled bars) and liver (dashed bars) of newborn and weaned rabbits. Data represent the mean (±standard error of the mean) of the expression ratio (normalized to newborn OE) carried out with 3 independent extractions. The significant differences in transcript level ratio are noted (a) for a comparison with newborn OE and (b) with weanling liver. $*P < 0.05$, $**P < 0.01$ (Pair Wise Fixed Reallocation Randomisation Test).

Table 2 Orthology analysis

Rabbit GSTs of the study		Orthology analysis			
			Highest BLAST scores		
Class	GenBank accession number	Class	Species	Enzymes	RefSeq protein ID
Alpha	M74528	Alpha	*Homo sapiens*	GSTA1	NP_665683.1
				GSTA5	NP_714543
				GSTA3	NP_000838
		Alpha	*Rattus norvegicus*	GSTA3	NP_113697
		Alpha	*Mus musculus*	Gsta3	NP_001070821
Mu	L23766	Mu	*Mus musculus*	Gstm2	GS
		Mu	*Rattus norvegicus*	GSTM2 (GSTM2-2)	NP_803175
		Mu	*Homo sapiens*	GSTM4 (GSTM4-4)	NP_000841
Pi	BI993585	Pi	*Homo sapiens*	GSTP1	NP_000843
Microsomal	AY050567	Microsomal	*Rattus norvegicus*	MGST3 predicted	ENSRNOT00000005719
			Mus musculus	Mgst3	NP_079845
			Homo sapiens	MGST3	NP_004519

Inparanoid algorithm was used to find orthologous GST isozymes (in rat, mouse, and human) corresponding to the rabbit GST mRNA of this study (Remm et al. 2001).

receptors (Thiebaud et al. 2013). Except for carboxylesterase expressed in the olfactory mucus, the demonstration of a role of xenobiotic-metabolizing enzymes in olfactory signal modulation involved intracellular enzymes, suggesting that odorants diffuse through the plasma membrane of OE cells. However, in all above studies, the stimuli were conventional odorants, that is, odorants which did not carry predisposed chemocommunicative value.

The understanding of perireceptor events surrounding ligand–receptor interactions have greatly benefited from insects pheromones. The automaticity and repeatability of insect behavior released by such biological signals has indeed constituted a unique model system to decipher the functional consequences of altering the biochemical perireceptor environment (Maïbèche-Coisne et al. 2004; Chertemps et al. 2012). Here, we used a mammalian model to explore

the catabolism of a pheromone in the OE. In the rabbit, the MP compares with insect pheromones in terms of spontaneous activity, functional clarity and, as tests are run on newborns, ease of handling. We investigated phase II metabolism of this pheromone because that process was otherwise suggested to be a major cause of odor signal termination in mammalian olfaction (Lazard et al. 1991; Tamura et al. 1998; Leclerc et al. 2002; Thiebaud et al. 2013).

Glutathione conjugation is a well-known pathway for the phase II biotransformation of aldehyde compounds in mammals and insects (Hayes et al. 2005). We demonstrated here that different odorants carrying an aldehyde function were efficiently conjugated to glutathione in newborn rabbit's OE, including the MP. As previously noted, certain aldehydes tested presented a spontaneous conjugation to glutathione (Testa and Krämer 2008), but their conjugation was strongly increased in presence of OE homogenate containing the metabolizing enzymes. Spontaneous conjugation is efficient during in vitro reaction because of the high concentration of the reactants and their higher reactivity in artificial media compared with the biological cellular environment. Among the aldehydes that are conjugated enzymatically, 3MB2 and cinnamaldehyde presented, respectively, significant versus nonsignificant activity on the pup sucking behavior, although 3MB2 provoked very weak responsiveness compared with the MP. In OE, these different activities did not impact the statistical significance of glutathione conjugation activities toward these aldehydes and between neonates and weaned rabbits. As expected, the glutathione conjugation in the OE was not specific to the MP. However, we interestingly observed a higher ratio neonates/weaned conjugation activity for the MP than for 3MB2 and cinnamaldehyde, meaning that olfactory catabolism process of the MP is of particular importance in newborns. Cinnamaldehyde presented a very similar variation profile to the MP even in the liver in which it appeared more metabolized at birth than after weaning. This could suggest a more important neonatal olfactory feature of this chemical than 3MB2. CDNB metabolism was also studied as non-aldehydic substrate (CDNB has been suggested to smell like almond in humans). To our knowledge, no olfactory behavioral test or electroolfactogram have been performed with this chemical, and its odor properties in animals are largely unknown. Nevertheless, it has been used in comparison with other odorants to study olfactory metabolism in different studies (Ben-Arie et al. 1993; Rogers et al. 1999; Whitby-Logan et al. 2004). Interestingly, here, the glutathione activity toward CDNB was clearly different from those observed toward aldehydes, both between tissues and along development. Opposite variations were even measured between MP and CDNB regarding activities in newborns and weanlings. Since CDNB probes a large spectrum of GST, the results suggest that, in OE, aldehydes glutathione conjugation probably involves GST isozymes with high affinity, which are therefore not or less involved in CDNB conjugation. This emphasizes also that the newborn OE definitely exhibits a high level of conjugation activity directed toward aldehydic odorants.

Glutathione conjugation clearly appears as a metabolic pathway for the MP in the OE. Thus, in accordance with its activity, the expression of GST in the rabbit OE was detected. The isoenzyme alpha, mu, Pi "like," and microsomal appeared strongly expressed. GSTs were previously widely characterized in rodent OE (Banger et al. 1993, 1994; Kudo et al. 2000) and a specific olfactory isoform was identified (Ben-Arie et al. 1993). Moreover, as demonstrated for olfactory receptors (Ressler et al. 1993), a zonal distribution of GST was observed throughout the mouse OE, suggesting a cooperation between metabolism and reception (Whitby-Logan et al. 2004). Here, we noted a higher level of expression of the studied GST in the OE versus the liver of newborn rabbits. There was also a higher expression of GST alpha in the OE as compared with the liver after weaning, and except GST Pi "like," all genes were underexpressed in the liver at birth compared with weaning. Thus, GST expression changes drastically during development; the enzymes in the liver appear to reach an "adult" level after weaning, whereas in the OE, the GST are expressed at the same level from birth to weaning. Such age-related variations have already been shown in other species. For example, Banger et al. (1993) demonstrated that in the rat OE, microsomal GST activity increases between postnatal days 3 and 84, with the same repartition in the liver for cytosolic GST. GST expression in the liver and intestine increases also during development to reach an adult level after weaning (Jang et al. 2001; Elbarbry and Alcorn 2009). In the rat OE, an increase in some GST isoforms (especially alpha and mu) occurs during the first postnatal days peaking at day 11 (weaning at day 21), with fewer changes in the respiratory epithelium (Krishna et al. 1994). In the red salmon, *Oncorhynchus nerka*, the developmental expression of a GST (soGST) in olfactory sensory neurons goes with neuronal growth, indicating a role of this GST in olfactory detection (Yanagi et al. 2004). Our orthology analysis suggests that the GST mRNA studied would correspond to the isosymes GSTA3, GSTM2, GSTP1, and MGST3. Interestingly, in mouse OE, Gstm2 shows an enriched expression (Yu et al. 2005). Besides, in mouse embryo (E14.5), a strong expression of Gstm2 was shown in OE by in situ mRNA analysis (Visel et al. 2004). At the same embryonic day of development, MGst3 exhibited also a strong expression, whereas Gsta3 was moderately expressed and no expression of GSTP1 reported. On the whole, review of the literature shows that correlations between species are difficult regarding both expression and substrate specificity of the GSTs. Anyway, in mammals, these developmental changes correspond to the prenatal and neonatal periods, when organisms are massively exposed to xenobiotic agents; therefore, during these periods, they need to establish metabolic barrier functions in the tissues most directly exposed to the environment or dedicated to detoxification.

Taken together, the present findings suggest that a high olfactory glutathione metabolism toward the MP is functional in newborn rabbits. During the nesting/suckling period, newborn rabbits are in principle minimally exposed to toxic volatile compounds arising from the soil of the burrow (or the nest box), the nest material, littermates, or the female and the matters she carries into the nest, especially milk. During the first 10-day period after birth, the pups' diet consists almost exclusively in milk, which contains a range of volatile components (more than 150 were identified), including the MP (Coureaud 2001; Schaal et al. 2003; Coureaud, Fortun-Lamothe, et al. 2008; Coureaud et al. 2010). As the MP appears to be a strong inducer of localization and oral grasping of nipples, its immediate detection is critical for pup survival; there is also a need to rapidly terminate its action when these responses are expressed. The active catabolism occurring in OE could therefore contribute to terminate the sensory impact of the MP by clearing it from the perireceptor space. These results concord with the relative absence of adaptation to the MP observed in rabbit pups: in a series of 15-s presentations repeated immediately after the end of each stimulation, newborn rabbits still strongly respond to the MP after more than 10 stimulations (i.e., >2.5 min; Coureaud 2001; Coureaud et al. in preparation).

As mentioned in the Introduction, such rapid peripheral deactivation of the MP may be essential in the species-typical structure of suckling in *Oryctolagus*: During the brief nursing episode, pups do indeed engage in several cycles of head searching-oral grasping motor actions triggered by the MP and swap from one to the same nipple or to another nipple every 15–20 s, without apparent preference (random choice depending on nipples proximity and availability in a context of strong competition), at least during the first nursing episodes (e.g. Hudson and Distel 1982; Bautista et al. 2005; Coureaud, Fortun-Lamothe, et al. 2008). To obtain enough milk, it is therefore necessary that the detection system of the MP, and possibly of other odor cues, remains highly responsive during the 5 min of nursing. Thus, after efficient nipple grasping occurs, the olfactory receptor(s) need to be made ready for a new cycle of MP activation and nipple searching. Additionally, a sensitivity to the MP remaining high during nursing may promote the learning of other odors carried by the maternal abdomen, which become novel significant cues for the newborn in addition to the MP (Coureaud, Moncomble, et al. 2006; Coureaud et al. 2010).

In conclusion, we present here a first set of findings on a potential perireceptor mechanism of chemosignal control and termination in a unique mammalian pheromonal model. The GST phase II appears as an effective metabolic pathway for the MP in the OE of the rabbit neonate. GST activity is particularly active against the MP (relative to other aldehydes) in the OE (relative to the liver). Moreover, this activity is higher in the milk-dependent pup relative to the weaned animal who does no more depend on milk and does no more respond to the MP by sucking action (Coureaud, Moncomble, et al. 2006; Coureaud, Rödel, et al. 2008); this suggests a specialized involvement of GST in the MP catabolism during early development. To more definitely establish the function of this olfactory metabolism in the detection of the MP, future experiments will have to counteract it. In particular, we will use inhibitors/competitors of GST to determine the ex vivo modulation of MP degradation over time and will assess *in vivo* the ensuing consequences on neonatal responsiveness to the MP and sucking success in the natural context of nursing. Further, it will be of highest interest to identify the GST in charge of the MP metabolism in the immediate surroundings of the olfactory receptors; obtaining such purified enzymes would allow characterizing the deactivation kinetics of the MP and other aldehydes and ultimately provide insight into the timing of the molecular processes underlying the regulation of a behavior that ensures survival of individual pups and the perenniality of a species.

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